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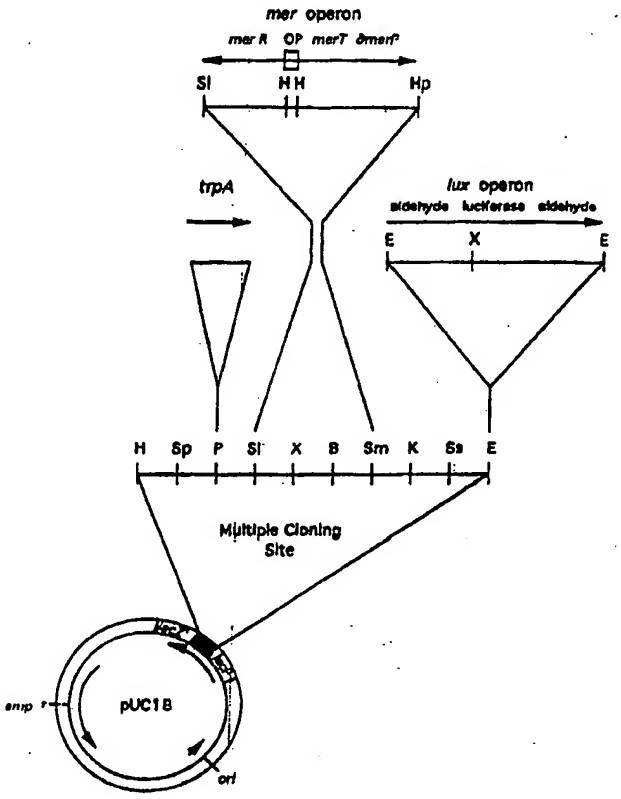
(54) Title: DEVICE FOR DETECTING AQUEOUS CONTAMINANTS

(57) Abstract

Device for detection of small quantities of a contaminant in water, comprising a light detecting means and a microorganism that emits significant detectable light only when exposed to a specific contaminant. Also, plasmid cassettes and host microorganisms containing such cassettes for use in the detection device.



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TITLE

DEVICE FOR DETECTING AQUEOUS CONTAMINANTS

BACKGROUND OF THE INVENTION

This invention pertains to a device for detection of small quantities of a contaminant in water, comprising a light detecting means and a microorganism that emits significant detectable light only when exposed to a specific contaminant.

To detect small quantities of contaminants, standard procedures are the use of atomic absorption spectrophotometry, ion chromatography, gas chromatography or mass spectrometry. These techniques require expensive equipment and high expertise, and cannot be done in a short period of time, e.g. in minutes.

It is known to use a bioluminescent test for genotoxic agents including mutagens, DNA-binding agents, DNA synthesis inhibitors and DNA intercalating agents as described by S. Ulitzer, 1986, Bioluminescence test for genotoxic agents, Methods in Enzymology, Vol. 133, pp. 264-276. This test is specific for these three classes of compounds. This test utilizes dark mutants of Photobacterium phosphoreum NRRL B-11177 or dark mutants of Photobacterium leiognathi P.f.-13. These bacteria have the genes for luminescence (lux operon), but do not express (transcribe and translate) these genes. After 1 to 8 h of exposure to a genotoxic agent, luminescence is induced in the dark cultures. The luminescence r sults from one of three mechanisms:

1) blocking the formation of a repressor of the lux operon; 2) inactivation of the repressor; or 3) changing of the physical configuration of the DNA.

Also, it is known to use a bacterial biosensor, "Microtox" made by Microbics Corporation, for detecting contaminants of an aqueous environment. "Microtox" consists of freeze-dried Photobacterium phosphoreum NRRL B-11177 that naturally have and express lux genes, yielding, under normal circumstances, strong light. The usefulness of the invention has been demonstrated by: 1) A.A. Bulich, 1986, Use of luminescent bacteria for determining toxicity in aquatic environments, in Aquatic Toxicology, ASTM STP 667, L.L. Marking and R.A. Kimerle (eds.), American Society for Testing and Materials, Philadelphia, PA, pp. 98-106; 2) A.A. Bulich, 1982, A practical and reliable method for monitoring the toxicity of aquatic samples, Proc. Biochem. 17:45-47; and 3) M.T. Elnabarawy, R.R., Robideau, and S.A. Beach, 1988, Comparison of three rapid toxicity test procedures: "Microtox", "Polytox", and activated sludge respiration inhibition, Toxicity Assessment: An International Journal 3:361-370. However, in the presence of any one or more pollutants, including metal ions (e.g. Hg^{2+}, Cu^{2+}, Cr^{2+}, Cd^{2+}) and organic compounds (e.g. sodium laural sulfate, formaldehyde, phenol, chloroform), luminescence is inhibited resulting in less and/or no light. This biosensor reacts to any circumstance that decreases the metabolic processes of the cell. As light production in the cell is tied to metabolism, damage to metabolic processes results in decreased light output. Consequently, this system is not pollutant specific. It can merely signal the presence of some material that adversely affects

metabolism.

Recently, since the present invention, excellent work has been published by Susan Frackman et al., Journal of Bacteriology, Oct. 1990, p. 5767-5773. This reference is incorporated herein by reference. It describes techniques for introducing lux genes into plasmids that are introduced into Escherichia coli. This publication does not encompass the inclusion into the plasmid of a regulatory element that is induced by a specific material. Nor does this publication disclose using inducible regulator/lux operon fusions in plasmids transformed into host bacteria as metal ion- or organic-specific biosensors. However, this reference has achieved the insertion Xenorhabdus luminescens lux gene fragments and the complete lux operon into plasmids that were then transformed into and expressed in Escherichia coli.

More recently, there has been published DE 3, 902, 902A (Gen lux, Aug. 2, 1990) which discloses that suitably equipped organisms react specifically to the presence of mercury by an increase in bioluminescence, that by making light in contrast to decreased luminescence reaction of prior art "Microtox" sensor systems. Specifically, this publication discloses a plasmid vector containing parts of an operon that can be induced by mercury, mer operon, linked to a lux gene complex from Vibrio harveyi; so that the presence of mercury ions stimulates the lux operon and therefore bioluminescence of the microorganisms. The utility of the microorganisms containing such plasmids is to introduce them into sewage treatment ponds, and so indicate the presence of mercury in the pond. This development is an excellent advance in biosensor detection of pollutants. However, superior results

are obtainable using preferred aspects of the present invention.

The use of lux genes as reporters of transcriptional activity is well documented in the published literature. A few examples of these uses are noted here.

J.E. Engebrecht, M. Simon, and M. Silverman, 1985, Measuring gene expression with light, Science, 227:1345-1347, first demonstrated that promoterless lux genes cloned from Vibrio fischeri, inserted into the transposon mini-Mu, could induce mutations by inactivation of a target gene-the resulting gene fusion produced light as a function of target gene expression. It was proposed that this system could be used to study the regulation of a variety of gene systems. This publication discloses cloning of a complete lux operon without its native promoter into Mini-Mu to create Mini-Mu lux and its use as a reporter of lac or ara gene transcriptional activity. It does not suggest or demonstrate fusion of the lux reporter to specific regulatory genes for chemical sensing purposes or for biosensor development.

O.A. Carmi, G.S.A.B. Stewart, S. Ulitzur, and J. Kuhn, 1987, Use of bacterial luciferase to establish a promoter pobe vehicle capable of nondestructive real-time analysis of gene expression in Bacillus spp., J. Bacteriol 169:2165-2170, report construction of a promoter probe vehicle allowing sensitive measurement of transcriptional activity from random genomic DNA fragment inserts. This plasmid allowed gene expression in either E. coli or Bacillus spp. to be measured as bioluminescence. This publication discloses use of V. fischeri lux fusions to follow gene expression during Bacillus sporulation differentiation processes, but does not disclose use

f lux gen fusions as a report r of specific chemicals in aqueous samples.

E.S. Rattray, J.I. Prosser, K. Killham, and L.A. Glover, 1990, Luminescence-based nonextractiv technique for in situ detection of Escherichia coli in soil, measures numbers of E. coli in liquid or soil by quantitative luminomitry. Constituative expression of V. fischeri lux operons allows detection of 10^2 to 6 X 10^3 cells ml^{-1} in water and soils, respectively. This publication discloses use of lux as a quantitative reporter of cell number, but not use of lux as a reporter of chemical concentration.

J.M.H. King, P.M. DiGrazia, B. Applegate, R. Burlage, J. Sanseverino, P. Dunbar, F. Larimer, and G.S. Sayler, 1990, Rapid, sensitive bioluminescent reporter technology for naphthalene exposure and biodegradation, Science 249:778-781, reports use of a transposon lux gene cassette from V. fischeri to generate bioluminescent reporters for naphthalene catabolism. The reporter biosensor is used for on-line process monitoring and control. The naphthalene degradation rate is monitored by measuring decreased luminescence resulting from decreased oxygen concentration during active metabolism of naphthalene, rather than naphthalene gene regulation. This publication discloses use of lux transcriptional fusions with catabolic genes for analysis of biodegradative microbial activity. The present invention presented in this patent measures chemical concentration by response of specific gene regulatory elements to the presence of a chemical directly controlling luminescence in the lux fusion biosensor.

SUMMARY

BRIEF DESCRIPTION F DRAWINGS

Fig. 1 Schematic representation of a lux cassette plasmid showing the original pUC18 cloning vector and representative sites and direction of transcription of the various elements (arrows) of the lux operon and a transcription terminator.

Fig. 2 Restriction map of lux operon from Xenorhabdus luminescens and the direction of transcription (arrow). Restriction endonuclease sites are abbreviated as follows: Bs, Bst E11; C, Cla 1; E, Eco R1; H, Hnd III, M, Mlu 1; S, Sca 1; X, Xba 1; B/Sa represents the joining of Bam H1 and Sau 3a cut DNA.

Fig. 3 Mercury genes used to engineer Hg^{2+} specific biosensors. A restriction map of mer regulatory element of RF mGN2-220. Arrows indicate direction of transcription.

Fig. 4 Mercury genes used to engineer Hg^{2+} specific biosensors. A schematic of 1.4Kb Sal I/Apa I mer fragment incorporated into the lux cassette plasmid.

Fig. 5 shows pCGLS201 relative light emission in a range of HgCl2 concentrations.

Fig. 6 shows immediate response of pCGLS201 in LE392 grown in LB + Amp and transferred at t=6h to LB + Amp with and without HgCl2.

Fig. 7 shows light emission of pCGLS201 in LE392 one hour after transfer at time indicated to media with and without HgCl2.

Fig. 8 shows pCGLS 206, 207 Relative light per cell in 0, 0.1 ug/ml HgCl2.

Fig. 9 is a table of examples of commercially available plasmid cloning vehicles.

Fig. 10 is a table of examples of suitable E. coli strains as host carriers.

Fig. 11 is a table of examples of suitable Bacillus and Pseudomonas strains to be used as host carriers.

Fig. 12 is a table of examples of known bioluminscent bacteria with lux systems suitable for use in biosensors.

Fig. 13 is a tabular summary of characterized metal systems.

Fig. 14 is a table of examples of possible organic contaminants sensed by biosensors.

Fig. 15 is a listing of recombinant mer plasmids derived from 1.4Kb mer fragment and lux cassette plasmid.

SUMMARY OF THE INVENTION

This invention is a device for detection of small quantities of an inorganic or organic contaminant in liquid or vapor water environment. It is capable of qualitatively and quantitatively detecting specific contaminants at low concentrations, in the parts per million (ppm) and billion (ppb) ranges. The invention utilizes the inherent sensitivity of certain biological systems to low concentrations of specific chemicals, coupled with the ability of certain biological systems to emit light.

Biosensors of this invention can be constructed for virtually any specific metal or organic compound. The invention provides a way to clone regulatory elements that control inducible response to specific chemicals from organisms resistant to the particular chemical. The regulatory

lement is fused to an optimized lux reporter operon during the cloning. Upon transformation into a suitabl host, a chemical-specific biosensor is constructed. It is possible to rapidly create a biosensor, even if the regulatory element of interest has not been characterized.

Biosensors generated by this process are used to rapidly detect, in 15 to 30 minutes, specific chemicals. The biosensor test is sensitive, selective, specific, nondestructive, and easy-to-use. Biosensors can be engineered for water-quality testing at consumer and industrial levels. Consumer tests include drinking water, both municipal and well water, and recreational waters. Industrial tests include municipal water works, well water, industrial process water, industrial supply and effluent waters, sewage treatment plant inflow and treated waste water, and environmental analysis of groundwater and soil. The tests are also applicable to feeds such as canned goods, frozen foods, and perishable products.

The test can be used by untrained personnel in the field, at industrial sites, or on the bench in analytical laboratories. The biosensor test is adaptable to spot tests, automated on-line continuous monitoring, and on-line process control.

The ultimate product of the present invention is a contaminant detection device containing a microorganism biosensor, or series of biosensors, that detect minute quantities of specific forms of chemicals in aqueous systems, when in the presence of such chemicals, the microorganism emits light.

These biosensors are made possible by the discovery of specifically engineered recombinant plasmid cloning vehicl s, that are transformed into competent single host c ll carrier microorganisms to

give the carrier contaminant specific bios nsing capability.

New r combinant plasmids of this inv ntion are engineered from known cloning vehicles (plasmids) to contain a promoterless lux operon, a chemical-specific regulatory gene, in some cases a Transcription Terminator, and a selectable antibiotic gene are engineered by known techniques. The lux operon is cloned into one end of a multiple cloning site (MCS) of the cloning vehicle, such as the Eco RI site in pUC 18. These new recombinant plasmids, when transformed into suitable competent host cells, "transform" the host cells such that very low or undetectable levels of light is emitted, since the luminescent genes are promoterless and hence are minimally expressed.

This new recombinant plasmid is ready for introduction of any of a variety of inducible regulatory elements consisting of a regulatory gene under control of its natural promoter/operator that is activated into expression only by the specific material to be monitored. Into the multicloning site of aforementioned plasmids, immediately upstream of the inserted lux operon of this invention is cloned an inducible regulatory element. The regulatory element is oriented such that the lux operon is under the control of the newly cloned regulatory element; the result of this cloning is commonly known as a "gene fusion". The regulatory element is activated into expression only by the specific material to be monitored; the lux operon is expressed only under the control of the activated regulatory element, producing substantial light.

Biosensors that monitor more than a single ion can be made. These comprise more than a singl

compound-specific promotor, in the same or different plasmids, in the same or different carrier microorganisms.

Another aspect of the invention is the host carrier microorganisms into the cytoplasm of which have been transferred plasmids of the present invention. Other aspects of the present invention are the methods of preparing and using the aforesaid plasmids, carrier organisms and detection devices of the invention.

The contaminant detection device contains biosensory carrier microorganisms, means for exposing these microorganisms to media to be tested, and means for detecting light emission from the microorganism.

DETAILED DESCRIPTION OF THE INVENTION

The recombinant plasmids of the present invention are prepared by cloning into a plasmid cloning vector, by known techniques, a promoterless bacterial lux operon and an inducible promoter that is activated by the specific material to be detected. The resultant recombinant plasmids are then transformed, by known means, into the cytoplasm of host microorganism competent for transformation, to form biosensor cells. Known methods, techniques, and procedures are substantially those found in molecular cloning and genetics guides such as T. Maniatis, E.F. Fritsch, and J. Sambrook, 1982, Molecular cloning; a laboratory manual, Cold Spring Harbor, N.Y. or Promega Protocols and Applications Guide, 2nd edition, 1991, Madison, WI. Enzymes, plasmids, and other materials used in this invention are typically available from commercial sources such as BRL or Promega, Madison, WI. Upon exposure to the specific material, for example mercury ions (Hg^{2+}), the regulatory gene

initiates expression of th lux op ron r sulting in light emission from the biosensor cells. The amount of light produced is a measure of th amount of mercury in the test system.

PLASMID

The plasmids, into which the specific regulatory element and the lux operon are cloned, is herein referred to as a "cloning vehicle". The cloning vehicle contains neither an inducible regulatory element specific to a particular contaminant nor a lux operon. It should have a multiple cloning site (MCS), or be modified to contain a MCS, receptive to cloning of these genes. A multiple cloning site is a small coding region of the plasmid that contains DNA sequences recognized by several specific restriction enzymes. These specific restriction enzymes cut the plasmid only once and cut only within this region, thereby making it possible to insert foreign DNA into a variety of different sites within the MCS.

The cloning vehicles are, in some cases, multicopy plasmids that individually generate a multiplicity of plasmids. Multicopy plasmids, such as pUC18 and pU19, can be obtained from commercial sources such as GIBCO/BRL, Gaithersberg, MD. A variety of other plasmid cloning vehicles, including low copy number plasmids, can be used for generating the recombinant plasmid (see for example Table 1).

Recombinant plasmids derived from pUC cloning vehicles also have genes for lac ? complementation, a modified lacZ gene, and the specific promoter for these genes (Plac). This feature is not essential to this invention. However, if this f ature is pr sent in the cloning vehicle used

for cloning, the regulatory elem nts and the lux genes must be inserted in an ori ntation pposite t that of the Plac promoter (Figure 1), in order t avoid possible non-specific expression of the lux operon by Plac.

The plasmid to be cloned desirably also contains an antibiotic resistance gene, such as an ampicillin or tetracycline resistance gene. This facilitates the selection from the general population of host cells those desired transformed host cells containing a recombinant plasmid rather than those host cells that are not transformed and hence do not contain a plasmid; only cells with plasmid are resistant to the antibiotic and therefore can grow. The total product from the cloning and transformation is simply subjected, by plating, to a solid agar medium containing the given antibiotic for which the transformed cells now carry resistance (e.g. 50 ug/ml ampicillin). Transformed cells will form colonies that are dimly luminescent.

HOST CARRIER MICROORGANISM

The host carrier microorganism can be any single cell organism that is or can be 1) made competent for transformation by the recombinant plasmid. The organism 2) must not contain any mechanism that would compete or interfere with the fused regulatory element/lux operons contaminant detection mechanism. Also, the organism 3) must not be significantly disabled or killed by contaminants in the media to be tested. Finally, the organism 4) cannot contain promoters that continuously activate the lux operon to a significant light-emitting degree or 5) that induce the lux operon to express significant luminescence in the presence f a

contaminant other than that which is to be detect d.

Thus the host carrier cell can be a bacterium, algae, fungi, yeast or mold in the preferr d construction, th host cell is a bacterium. E. coli is the most preferred host carrier because it is the best characterized and most easily manipulated system in terms of it's genetics and the range of molecular techniques that have been developed for this host carrier. A listing of some of the useful E. coli strains is given in Table 2.

However, not all cloned genes are compatible with the E. coli carrier host system. Many genes from other organisms such as Pseudomonas sp. are either not maintained or are poorly expressed, if there is any expression at all, when cloned into E. coli. Therefore, other plasmid systems and compatible host systems are also utilized for this invention, including systems based on host carriers such as Pseudomonas sp. or Bacillus sp. (see Table 3).

Substantially all of the host organisms that are effective carriers of the recombinant DNA plasmids of this invention require treatment to render them capable of transformation. This treatment modifies the host cell organisms so that they are "competent" to take up exogenous DNA across their cell walls and into their cytoplasm. Bacterial cells are made competent by chemical treatment of mid-exponential growth phase cells commonly 1) with high concentrations of $CaCl_2$ or RbCl, or 2) by washing and resuspending cells in low ionic strength buffers to produce electrotransformable cells.

The transformation of host carrier cells is carried out using known techniques by mixing competent or electrotransformable cells with recombinant plasmid DNA. The chemically tr ated cell/DNA mixture is heat

shocked briefly, while th electrotransformable cell/DNA mixture is treated with high voltag electrical pulses (electrop rati n). Tr ated c ll suspensions are allowed to recover briefly and then are diluted and plated on suitable growth media such as Luria Broth (LB) agar plates containing an antibiotic such as ampicillin (e.g. for pUC18). Only transformed bacteria will be resistant to the antibiotic and will grow. If algae, fungi, or molds are to be transformed, a third transformation technique may be employed to facilitate introduction of lux cassette plasmid DNA into cells, the Biolistic Partical Delivery System (E. I. du Pont de Nemours & Co., Wilmington, DE.)

LUX OPERON

The bacterial lux operon codes for the five structural genes required for luminescence; luxA and luxB encode subunits of bacterial luciferase while luxC, luxD, and luxE encode a fatty acid reductase complex. The enzyme bacterial luciferase requires both oxygen and a long chain aldehyde, (provided by the host cell), to produce light.

While the lux operon from Xenorhabdus luminescens is preferred, functional bacterial lux operons can also be obtained from a number of marine and terrestrial bacteria (Table 4). Prior art shows that useful lux operons have been cloned from Vibrio harveyi (see for example DE 3, 902, 902A) and Vibrio fischeri (see example J.M.H. King, P.M. DiGrazia, B. Applegate, R.Burlage, J.Sanseverino, P.Dunbar, F.Larimer, G.S.Sayler, 1990, Rapid, sensitive, bioluminescent reporter technology for napthelene exposure and biodegradation, Science, 249:778-781 or E.A.S.Rattray, J.I.Prosser, K.Killham, and L.A.Glover,

1990, Luminescenc -bas d nonextractive technique for in situ det ction of Escherichia coli in soil, Appl. Environ. Microbiol. 56:3368-3374. As already not d, the preferred lux operon is from Xenorhabdus luminescens. These bacteria are carried by the nematode Heterorhabditis bacteriophorce.

The X. luminescens lux operon is preferred because it gives off high intensity luminescence. It is also possible to eliminate the natural lux operon promoter and so there is minimal expression of the lux operon, and hence background luminescence can be very low. Equally important, X. luminescens luciferase performs well at elevated temperatures, up to 45°C. In contrast, luciferase from V. harveyi and V. fischeri rapidly become inactive at temperatures above 25°C (see example R.Szittner and E.Meighen, 1990, Nucleotide sequence, expression and properties of luciferase coded by lux genes from a terrestrial bacterium, J. Biol. Chem. 25:16581-16587).

The X. luminescens lux operon is preferred because the cloned gene system is complete. When a plasmid containing this operon is transformed into a suitable host cell, all coding elements necessary to produce light are encoded by either the lux operon DNA or are provided by the host cell. There is no need for addition of the aldehyde substrate typically required, for example, when Vibrio harveyi lux genes are cloned, or when only the genes for luciferase, luxA and luxB, from V. harveyi or V. fischeri are cloned.

Another important reason for our preference for the lux operon from X. luminescens is that this operon has been cloned into pUC18, and the cloned operon has been partially characterized (Frackman et. al). The lux op ron DNA is found on an 11 Kb ins rt

in plasmid pCGLS1 (see Figure 1). Prior art by S. Frackman dem nstrates that EcoR1 restriction nzyme digestion of pCGLS1 generates a fragment f about 6.9 Kb. This fragment appears to lack its natural promoter region and contains only the structural genes of the lux operon. When this fragment is religated into pUC18 and transformed into a suitable E. coli host using known techniques, the transformed cells are observed to be relatively free of promoter activity; that is, when inserted into the MCS or pU18 in the proper orientation opposite to that of the Plac promoter, the clones are dim and produce little light. It is desirable to minimize background light to maximize the signal to noise ratio of the biosensors and hence to enhance the sensitivity of the biosensor system.

REGULATORY GENE LUX CASSETTE CLONING VEHICLE

This invention incorporates the engineering of new recombinant plasmids from known cloning vehicles that contain promoterless lux operons. These new recombinant plasmids are known as "regulatory gene lux cassette cloning vehicles" or simply "lux cassette plasmid". Such new recombinant plasmids are used to isolate and clone the inducible regulatory elements of interest. Upon introduction by ligation of the regulatory element of interest into the lux cassette plasmid, followed by transformation of this recombinant plasmid into a suitable host carrier, a unique biosensor for a particular material is formed.

The EcoR1 fragment of the pCGLS1 lux operon, containing substantially promoterless lux structural genes, is cloned into one end of the MCS of the cloning v hicle by known techniques (see for example Maniatis t. al. and Frackman et. al.). Specifically,

1 Mu g of preferably CsCl purified pCGLS1 DNA is digested with the sit -sp cific restriction enzyme, EcoR1, using known techniques. The digested pCGLS1 DNA is s parated into component fragments by horizontal electrophoresis in an agarose gel 0.8% to 1% in 1X TBE buffer. A DNA fragment corresponding to the size of 6.9 Kb is identified and excised from the gel and eluted by known techniques such as extraction with glass milk (Biol01, Inc. La Jolla, CA) or a freeze-squeeze method; pUC18 is similarly digested with EcoR1 to linearize the circular plasmid and to prepare the plasmid for incorporation of the lux operon fragment. The eluted fragment is mixed with the linearized pUC18 plasmid, and upon addition of T4 DNA ligase, buffer, and after appropriate incubation conditions (Maniatis et. al.), the EcoR1 DNA fragment containing the structural lux genes is incorporated (ligated) into the plasmid (see Figure 1). This new recombinant plasmid is designated pCGLS200.

Desirably, this cloned lux operon is selected to contain the full content of genes luxC, luxD, luxA, luxB, and luxE. For best results, this operon is cloned into the EcoR1 MCS of pUC18 proximal to Plac. As previously discussed, it is essential that the operon be oriented such that the 5'-transcriptional orientation of the operon is opposite to the 5'-transcriptional orientation of Plac. In the recombinant plasmid pCGLS200, an Xbal digestion of the plasmid will yield two bands when the products of the digestion are electrophoresed as previously described in an agarose gel. A clone with the correct lux operon orientation will generate fragments of about 2.5 Kb and 7.2 Kb, while the incorrect orientation will generate fragments of about 4.5 Kb and 5.2Kb (see Figure 1).

It is possible to further reduce the background luminescence of this regulatory gene lux cassette cloning vehicle by removing material from the upstream, 5'-end of the lux operon. This effect is achieved by digesting pCGLS200 with Kpn1, which results in a linearized plasmid cut in the MCS just upstream of the lux operon (Figure 1).

The linearized plasmid is treated by known techniques with Nuclease Ba131 for 2, 3, 4, 5, and 6 minutes. Nuclease Ba131 cleaves duplex DNA exonucleolytically from both ends, producing successively shortend strands; cleavage results in mostly blunt ends. Only the 5' end of the lux operon is protected by plasmid DNA. The loss of the plasmid DNA that is also being deleted is of no consequence to this invention, as the cut-down lux operon is ultimately removed from this modified plasmid material and only the downsized lux operon is religated into new whole pUC18.

At the times indicated, the digestions are terminated by heating using known techniques. The Nuclease Ba131 digestions are sized on agarose gels and those digestions that yield deletions of approximately 500 bp are selected for further study. The appropriate digestions are then precipitated with ethanol and resuspended in 10 mM Tris, 1 mM EDTA, pH 8.0 (TE) using known methodology.

Nuclease Ba131 deletions destroy the Kpn 1 site at which the plasmid was originally linearized. Therefore, in order to circularize the plasmid, molecular linkers with appropriate restriction sites are ligated to the plasmid. The appropriate Nuclease Ba131 digestions are prepared for blunt end ligation of phosphorylated molecular linkers by any one of three ways: 1) no further treatment; 2) treatment with

Mung Bean Nucl as (an exonucl as that pr cesses single strand nds producing blunt ends; or 3) treatment of the ends with large fragment of DNA polymerase I (Klenow fragment) plus deoxyribotrinucleotides to fill in overhangs and thus producing blunt ends.

Phosphorylated Sst1 or Kpn1 molecular linkers are then ligated to the Nuclease Ba131 digestions with T4 DNA ligase. Modified ligated plasmids are cut with the appropriate restriction enzyme, Sst1 or Kpn1 in order to linearize the plasmid and to eliminate concatamers of linkers formed during ligation. Unicorporated linkers are removed by ethanol precipitation or spin column treatment using known techniques.

The clean, linearized plasmids, are circularized by ligation, then transformed into a suitable host, and dim or dark colonies are selected. Two of these are picked and designated pCGLS202 (from ligation with Sst1 linkers) and pCGLS203 (from ligation with Kpn1 linkers).

Intact regulatory gene lux cassette cloning vehicles are regenerated by removing the modified lux operon inserts from pCGLS202 by double digestion with EcoR1/Sst1, and from pCGLS203 by double digestion with EcoR1/Kpn1. The respective lux inserts are purified by electrophoresis on agarose gels, followed by excision and purification of the appropriate bands, as described above. Directed ligation into new pUC18 is achieved by doubly digesting the new pUC18 plasmid with either EcoR1/Sst1 or EcoR1/Kpn1, followed by standard ligation of the purified lux inserts into the appropriate linearized plasmid. The resulting plasmids are designated pCGLS204 and pCGLS205, resp ctiv ly (se Figure 3). Thes plasmids are r ady

for intr duction f any of a variety of inducible r gulatory lements.

In some combinations of the abov d scrib d lux plasmid cassette, and with certain host bacteria, background luminescence may still be excessive. In such cases, it may be desirable to insert into the lux plasmid cassette trpA Transcription Terminator (Pharmacia LKB BioTechnology, NJ) upstream of the lux operon and upstream of the intended site of cloning of a regulatory element, such as at the Pst1 site in the MCS. The Transcription Terminator is modified by addition of Pst1 phosphorylated linkers by ligation with T4 DNA ligase. The product of the ligation is cut with Pst1 using known standard conditions. The ligated product is purified by precipitation with ethanol or by spin column. Lux cassette plasmids, pCGLS204 and pCGLS205 are also cut with Pst1 and the Transcription Terminator/linker product is ligated into the plasmid as previously described. After transformation into suitable competent host carriers, transformed host cells are selected randomly from those that grow on LB plus ampicillin plates. Clones with Transcription Terminator in the proper orientation to the lux operon will be very dim or dark. The presence and confirmation of the proper orientation of the Transcription Terminator can be achieved by subcloning the MCS containing the Transcription Terminator into a M13 sequencing system using known techniques.

This Transcription Terminator incorporated into the lux cassette plasmid will prevent transcription of the lux operon in the absence of expression from a cloned regulatory gene (see Figure 1,3). By this technique, clones with exceptionally low backgr und luminesc nc can be prepar d.

REGULATORY ELEMENTS

The inducible regulat ry elements are taken from plasmids or from genonetic DNA in bacterial strains that are resistant to attack by the specific contaminant to be monitored. This gene and its promoter/operator functions in its parent bacteria to initiate protective anticontaminant activity by the bacteria upon exposure to the specific contaminant.

Many bacteria are known to be resistant to specific toxic materials. For example, some Serratia sp. are known to be resistant to Hg^{2+} ions. Other bacteria are resistant to specific contaminant materials, either inorganic metal ions or organic compounds; many of these materials are water soluble to some extent and examples are listed in Table 5.

Many other such resistant bacteria exist. Of particular interest for the products of the present inventions are bacteria that can provide regulatory elements specifically initiated by metals such as mercury, lead, cadmium, and chromium metal ions or by organics such as benzene, phenol, and PCBs. These bacteria can be isolated from aqueous areas, soils, and sediments known to contain the specific contaminant. The isolated bacterial strains can be propagated and maintained as a permanent source of plasmids containing the desired inducible resistance operon. Alternatively, the desired resistance operon containing the regulatory element can be maintained by taking from the resistant cells appropriate chromosomal DNA or plasmids, and cloning the genes for the resistances into plasmid cloning vehicles containing no promoter operon responsive to exposure to the contaminants; the regulatory genes for these resistances also can be cloned directly into the lux

cass tt plasmid.

Plasmids c ntaining inducible regulat ry elements and r sistance genes can be maintained and propagated in appropriate host cells using standard techniques. These plasmids can also be extracted from the host bacteria, purified, and stored in a frozen state using known techniques.

CONTAMINANT DETECTION DEVICE

The contaminant detecting device comprises biosensory cells, means for exposing these biosensors to the media to be tested, and means for detecting light emissions from the biosensors. Desirably, the means for detecting light will measure intensity of light as a function of concentration of the contaminant. The means for detecting light can be such that light can be detected by the eye, photographically, or electronically such as in combination with a metering device or a computer.

The light detecting means associated with the detection devices of the present invention can be of several types, depending on the method of use.

A film type detection means is useful for qualitative measurements. This means comprises a "Polaroid" (Polaroid Corporation) instant film, such as Type 667, that responds to the luminescence of a positive response from biosensor cells. An example of this film system has a film backing with an opaque block mounted above the film plane. Several vials containing one or more aqueous suspensions of biosensors are inserted into holes in the block so that their bases are exposed to the film. As many as 20 test vials are normally used. After the vials are placed in the block, a cover over the vials and block seals light from top side of the vials to th film.

Samples, aqu ous or gas ous, to be tested are injected at kn wn volumes into the vials, containing known v lumes or concentrations of biosensor microorganism cells. Aft r exp sure of the film to any luminescenc from the vials are removed by a plate between the vials and the film, the film is developed and the intensity of spots from bioluminescence are noted. The presence, clarity and brightness of any spots shows whether the particular contaminants are present in the tested samples and indicate their concentration.

One photomultiplier portable unit consists of a photomultiplier tube such as a Hamamatsu Corporation 1894 lead-on tube, which provides a strong response in the 500 NM range of luminescent output. The amplifier and high voltage power supply are battery powered for portability. This device is used to test samples loaded into a light-tight chamber. A data acquisition/computer system can be used to automate the sample logging process.

Another photomultiplier unit primarily for laboratory use consists of a photomultiplier type, such as a Hamamatsu R363 side-on tube which exhibits exceptionally flat response across the range of the light output of the biosensor cells. The system has a light-tight chamber for discrete testing of samples and a digital panel output meter. The photomultiplier amplifier is designed around an Analog Devices electrometer amplifier (an AD515). The electrometer amplifier is operated as a current-to-voltage converter using a switched series of high-value resistors (100 Kohm to 100 Mohm). In addition, noise damping is included by wiring manually switched low-value capaciters in parallel with the gain resistors. The output of the amplifier is calibrated

with a calibrated, industry-standard amplifi r (Pacific Photometrics Model 110 Photomet r) using th sam phot multipli r tube with a 4.5 digit readout voltmeter. Agreement between the tw amplifiers is within the precision and repeatability of the light source. A data acquisition/computer system can be used to automate the sample logging process.

The photodiode photometer is designed primarily for field use. The instrument is built using an instrumentation amplifier and a Hamamatsu photodiode subunit. This Hamamatsu HC220-01 has an integral fixed-gain amplifier with optional external offset adjustment. The sensitivity is 0.8 V/nW at the peak wavelength sensitivity at 720 nm. The instrumentation amplifier provides additional gain which is adjustable from a factor of 15 to 30,0000,(?) and greatly reduces noise at high gain. The readout is a digital voltmeter (DVM).

This photodiode photometer readily detects the output of a laboratory standard light source constructed from $^{14}CO_2$ in scintillation flour and sealed in a glass ampule, a precision of +2% is obtained and is limited primarily by geometric effects as the standard light source is much smaller than the sample vial.

The sensitivity and linearity of these photometers is determined by comparing the response of the photodiode instrument with the laboratory-standard photomultiplier photometer using bioluminescent cultures. The results of this comparison are shown in Table X and Figures X and XX. The photodiode photometer is fully capable of accurately quantifying bioluminescence of bacterial cultures. When fully derepressed bacteria are used, the output of the solid-state photom ter becomes nonlin ar, indicating

that the sensor is saturated (see Figure XX).

The biosensor cells of the pr sent invention are prepar d by transforming with such lux cassette plasmids, th host microorganisms. To prepare the detection device the resultant carrier microorganisms are put into distilled water at a predetermined microorganism concentration, and the aqueous microorganism suspension is then associated with means for exposing the microorganism to the media to be tested and the means for detecting a bioluminescence signal.

In use, the aqueous media to be tested for the presence of the specific material is introduced into the aqueous biosensor bacteria suspension. If the tested material contains the specific material, the regulatory elements will stimulate the lux operon transcription and translation of in the cassette, causing luminescence. The light thus emitted is sensed by bioluminescence detecting means, thereby expressing the positive result that the specific material is present in the aqueous media being tested. The intensity of the bioluminescence can be used to indicate the concentration of the specific material.

The inducible regulatory gene that is cloned into the plasmid base of the lux cassette plasmid is sensitive to one or more specific contaminant materials. When exposed to such material, normally metal ions or organic materials in an aqueous medium the regulatory element reacts to the material to signal expression from the lux operon that has also been cloned into the plasmid.

EXAMPLE 1: MERCURY BIOSENSOR

Biosensors for mercury (Hg^{2+}) are based on the regulatory gene merR from the mer resistance

operon of a Serratia sp., which was characteriz d by G. Nucifora, L. Chu, S. Silv r, and T. K. Misra, 1989, Mercury operon regulation by the merR gene of the organomercurial resistance system of plasmid pDU1358, J. Bacteriol. 171:4241-4247. MerR genes are obtained from S. Silver, University of Illinois, IL in the form of a clone in the sequencing M13 phage, mGN2-220 or as a plasmid such as pDU1358 or pGN110. This phage has the following insert from pDU1358: merR, operator/promoter, merT, merP, merA. (Figure 3).

The mercury biosensor is designed to contain the following portions of the mGN2-220 mer insert: merR, operator (O)/promoter (P), merT, merP. The protocols followed to achieve this construct are substantially those found in Maniatis et. al. and the Promega Applications Manual cited above.

First, double stranded replicative form of mGN2-220 is prepared. 100 ul of phage stock (from S. Silver) is added to a 1:100 dilution into 500 ml Luria Broth (LB) of an overnight culture of E. coli DH5?F'I^q grown in 3 ml of (LB) at 37°C. This culture is shaken vigorously at 37°C for 6 to 8 hours. The culture is centrifuged at 10,000 xg for 10 minutes to pellet the E. coli; the supernatant containing phage is discarded.

RF, which is amplified within the cells in the pellet, is obtained by a plasmid extraction procedure using known techniques. The pellet is resuspended in 25 ml of 20% sucrose, 100 mM Tris-HCl, pH 8.0, 20 mM EDTA (Solution 1) plus 2 mg/ml lysozyme suspended in 25 ml of the Solution 1. This is incubated at room temperature for 20 minutes. 100 ml of freshly pr pared 1% sodium dodecyl sulfate (SDS), 0.2 M NaOH (Solution 2) is add d to lyse the c lls;

th sample is incubated for an additional 30 minutes at room t mperature. 80 ml of ice-code 3M potassium acetate, pH 4.0 (Solution 3) is added to precipitate genomic DNA. After incubation for 1 hours at -20°C, the material is centrifuged at 10,000 xg for 30 minutes at 4°C; the supernatant containing the RF is poured through cheesecloth into a fresh centrifuge bottle. An equal volume of cold isopropanol is added, and mixed, after 10 minutes at room temperature, the sample is again centrifuged at 10,000 xg for 30 minutes to pellet the RF. The pellet is gently rinsed with 95% ethanol and then drained at least 15 minutes at room temperature to dry. Finally the RF DNA is resuspended in 4 ml of 10 mM Tris-HCl, pH 8.0, 1 mM EDTA (TE).

The RF is then purified by banding in CsCl by isopycnic density centrifugation. To the 4 ml sample is added precisely 4 g of CsCl plus 400 ul of a 10 mg/ml ethidium bromide solution. The sample is loaded into appropriate sample tubes for centrifugation in a Beckman VT165.1 or Sorvall TV-1665 vertical centrifuge rotor, or equivalent rotor, for 16 hours at 55,000 rpm at 15°C. Two bands are observed-the lower RF band is in the middle of the tube, and is removed with a syringe through an 18 gauge needle using known techniques. The ethidium bromide is removed from the RF DNA by extraction with an equal volume of isopropanol saturated with TE and NaCl; the upper layer (isopropanol) turns pink and is discarded. Isopropanol extraction is repeated until color is no longer observed in the upper phase; two additional extractions with isopropanol are then completed. The CsCl is removed from the sample by either: 1) dialysis against 3 to 4 changes of TE for 24 hours, followed by addition of 0.1 volume of 3M

sodium acetat and precipitation with two volumes of ethanol at 4°C r at room temperature, or 2) by pr cipitating the RF DNA after increasing the volum three fold with distilled water and adding sixfold ethanol at 4°C or room temperature, resuspending the precipitated DNA in 5 ml of TE, then repeating the precipitation by addition of 0.1 volume of 3M sodium acetate and precipitation with two volumes of ethanol at 4°C or at room temperature. The DNA is brought up in 1 to 5 ml of TE and the DNA concentration determined by spectroscopy using known techniques.

RF is double digested with restriction endonucleases to obtain the desired mer operon fragment of approximately 1.4 Kb: merR, O/P, merT, merP. To 1 ug of DNA is added 1 ul of Hpa 1 and 1ul of Sal 1, 2 ul of 10X buffer ("GIBCO/BRL REact4"), and distilled water to make a final volume of 20 ul. The sample is incubated for 1 hour at 37°C. The completeness of the linearization/digestion is determined by electrophoresis in an 0.8% agarose gel in 1X TBE.

The 1.4 Kb fragment is then purified by electrophoresis in a preparative 0.8% agarose gel. The 1.4 Kb band is excised and extracted by freeze-squeeze using known techniques. The DNA is precipitated by addition of 0.1 volume of 3 M sodium acetate and 2 volumes of ethanol. The DNA is resuspended in 1 ml of TE and the concentration determined by spectroscopy.

The 1.4 Kb mer operon fragment is next incorporated into appropriate lux cassette plasmids, such as pCGLS200, pCGLS204, or pCGLS205. The lux cassette plasmid is doubly cut with Sal 1 and Sma 1 using known techniques. 5 ug of the appropriate lux cassette plasmid is digest d with 1 ul of Sal 1

("GIBCO/BRL"), 2 ul f "REact" 10, and th balance distill d water to make 20 ul total volume, for 1 hour at 37°C. Th linearized lux cassette plasmid is precipitated with ethanol as described above, and resuspended in 17 ul of water. To this is added 2 ul of Sma 1 and 1 ul of "REact" 4, and the DNA is digested for 1 hour at 37°C. Buffer and the small MCS fragment that is produced by digestion is removed by spin column (Select D-50; 5'-3', Inc., Boulder, CO) using known techniques. The eluted DNA is precipitated with ethanol as described and resuspended in 10 ul of distilled water.

This lux cassette plasmid DNA is now ready to incorporate by ligation, in a directed fashion, the 1.4 Kb mer DNA. The blunt ended Hpa 1 and Sma 1 sites will specifically ligate, and the sticky end Sal 1 sites will specifically ligate. The transcriptional orientation of the mer DNA will be in the same direction as the transcriptional orientation of the lux operon DNA; the result will be a transcriptional fusion of mer R, O/P, mer T, mer P and lux (see Figure 3).

The ligation is achieved by mixing 5 ul of the mer 1.4 Kb fragment, 2 ul of pCGLS200, pCGLS204, or pCGLS205, 5 ul of 5X ligase buffer, 1 ul of T4 DNA ligase ("GIBCO/BRL"), at 4°C for 4 to 24 hours. Figure 15 lists representative recombinant mer fusion plasmids of this invention generated from the 1.4 Kb mer fragment and lux cassette plasmids.

TABLE 6: Recombinant mer plasmids derived from 1.4 Kb mer fragment and lux cassette plasmid.

lux cassette plasmid	Recombinant mer plasmid	Comments
pCGLS200	pCGLS201	moderate background luminescence
pCGLS204	pCGLS206	dim background luminescence
pCGLS205	pCGLS207	very dim background luminescence

The ligation mixture is added to 0.2 ml of competent E. coli such as strain LE392 or strain HB101 (competent cells are prepared as previously described), and the mixture is heat shocked and 0.02 ml and 1.8 ml plated onto LB plus ampicillin plates using known techniques. The plates are incubated overnight at 35°C. Colonies are checked for low level of light production and for a luminescent response to mercury; those clones that meet these criteria are biosensors for mercury.

Luminescence of the biosensors is measured on a Pacific Photometrics Lab Photometer or amplifier/voltmeter with output from a photomultiplier tube or photodiode photometer as previously described or equivalent. Cell density is reported as optical density (OD) at 600 nm. Relative background luminescence levels of representative clones are summarized in Figure 15. The luminescent response of pCGLS201 [LE392] (plasmid pCGLS201 transformed into E. coli LE392) during growth with and without 0.025 and

0.1 ug/ml Hg^{2+} is shown in Figure 5. A measurable response of about 1.5 fold over background is seen with 0.025 ug/ml Hg^{2+}, a 17 fold response over background is observed.

Rapid response tests to Hg2+ demonstrate that these biosensors are sensitive and responsive (see Figures 6 and 7). The kinetics of the pCGLS201 biosensor to 0.1 ug Hg2+ shows the luminescent response for 6 hours mid-exponential phase growth cells is easily measurable within 15 minutes, is 80% of maximum within 30 minutes, and is essentially complete within 60 minutes (Figure 5). One hour rapid response to Hg2+ of cells taken at 2, 4, 6, and 8 hours of growth indicates that 6 to 8 hour cells are desirable when E. coli 1.E. 392 is the host carrier.

When pCGLS205 and pCGLS206, with even lower background luminescence levels than pCGLS201, are used to generate biosensors by transformation of the recombinant plasmid into E. coli LE392, the luminescent response during growth is even more dramatic (see Figure 8). It is expected that upon insertion of the Transcriptional Terminator, the response normally will be even greater. A variety of E. coli host carriers have been tested with similar results to those reported immediately above.

Other mercury biosensors with different sensitivities are generated by incorporation of different amounts of the detoxification genes of the mer operon. A construct with merR, O/P, merT, merP, merA is resistant to mercury and therefore has a broader range of response. Fusion of merB into any of the constructs mentioned will generate a sensor that is responsive to both inorganic and organic mercury compounds.

Additionally, mercury resistance genes from different microorganisms have different responses to mercury, and the regulatory elements respond to different levels of mercury. Thereby, a variety of mercury biosensors are created for applications that require low or high sensitivity and narrow or broad range of response.

EXAMPLE 2: OTHER METAL BIOSENSORS

Other metal biosensors are engineered in a fashion substantially similar to the mercury biosensor. Genes for incucible resistance systems or natural plasmids, or genomic genes that are known and have been previously characterized, or genes from natural plasmids or genomes that have been cloned into standard cloning vehicles, are used as sources of positive regulatory elements (see Figure 13 for examples of known resistances). The regulatory elements from these genes are isolated and introduced into lux cassette plasmids of this invention substantially as described for the mercury regulatory element to generate biosensors.

Other metal resistances are known but not cloned, or the location of the genes on natural plasmids or in the genome are not yet identified. An example of this type of inducibly resistant system is lead. Lead resistant microorganisms are isolated from water, soils, and sediments on solid media such as LB supplemented with various levels of lead (0.1 to 10 mM). Genomic DNA or plasmid DNA is shotgun cloned, using standard techniques, into lux cassette plasmids. The DNA is partially digested with a restriction endonuclease such as Sau3A, and 10 to 15 Kb fragments are ligated into a lux cassette plasmid. The recombinant plasmids are transformed into any of a

variety of suitable carrier hosts, which are plated nto LB plus ampicillin plates as previ usly described. Lead biosensor clon s are select d that substantially lumin sce only when lead is present. As with the mercury sensor, sensors with different sensitivities and range of response can be generated from this sensor by eliminating nonessential portions, or adding and keeping other portions, of the resistance operon.

EXAMPLE 3: ORGANIC BIOSENSORS

In similar fashion as that described in Example 1 and Example 2, biosensors responsive to specific organic compounds can be engineered. A wide range of microorganisms are capable of degrading specific organic chemicals and are used as a source of genes for engineering specific biosensors. A list of some of the organic compounds that biosensors are made for is given in Figure 14.

EXAMPLE 4: OTHER HOST CARRIERS

Host carrier systems are *E. coli*, *Pseudomonas* sp., *Bacillus* sp., and any of a variety of other known bacteria, algae, fungi, and molds. In most cases, selection of the host carrier is directed by compatibility of transcription of the regulatory element and other genes of interest. This is generally based on the particular standard cloning vehicle the *lux* cassette plasmid is generated from, and the general compatibility of a particular DNA with a particular host.

CLAIMS:

1. A device for detection of a contaminant in water comprising:

(i) means for detecting luminescence; and

(ii) microorganisms to be exposed to the sample to be tested, said microorganisms containing a plasmid vector comprising;

(a) an inducible regulatory gene that is activated by exposure to the specific material for which the detection device is designed, and

(b) a bacterial, substantially promoterless, lux operon that expresses light when induced by said regulatory gene.

2. A device in accordance with Claim 1 wherein said regulatory gene and lux operon are part of a plasmid cassette, with the regulatory gene being located upstream of the lux gene operon.

3. A device in accordance with Claim 2 wherein said lux operon is taken from Xenorhabdus luminescens.

4. A device in accordance with Claim 2 and 3 wherein said luciferase gene operon complex comprises luxC, luxD, luxA, luxB and luxE g nes.

5. A devic in accordanc with Claim 3 wherein said plasmid cass tt contains substantially no X. lumin scens pr moter.

6. A device in accordance with Claim 3 wherein said luxA and luxB genes are transcribed 5' to 3'.

7. A device in accordance with Claim 6 comprising genes that biosynthesize fatty acid reductase complex when located on both the left and right sides of the luxA and luxB genes.

8. A device in accordance with Claim 2 comprising an antibiotic resistance gene.

9. A device in accordance with Claim 2 comprising a transcriptional terminator located upstream of both the regulatory gene and the lux operon.

10. A recombinant plasmid comprising an inducible regulatory gene that is activated by exposure to a specific material and a bacterial, substantially promoterless, lux operon that expresses light when induced by said regulatory gene.

11. A recombinant plasmid in accordance with Claim 10 wherein the regulatory gene is located upstream of the lux gene operon.

12. A recombinant plasmid in accordance with Claim 10 wherein said lux operon is taken from Xenorhabdus luminescens.

13. A recombinant plasmid in accordance with Claims 11 and 12 wherein said luciferase gene operon complex comprises luxC, luxD, luxA, luxB and luxE genes.

14. A recombinant plasmid in accordance with Claim 12 wherein said plasmid cassette contains substantially no x. luminescens promoter.

15. A recombinant plasmid in accordance with Claim 14 wherein said luxA and luxB genes are transcribed 5' to 3'.

16. A recombinant plasmid in accordance with Claim 15 comprising genes that biosynthesize fatty acid reductase complex when located on both the left and right sides of the luxA and luxB genes.

17. A recombinant plasmid in accordance with Claim 11 comprising an antibiotic resistance gene.

18. A recombinant plasmid in accordance with Claim 17 comprising a Transcriptional Terminator located upstream of both the regulatory gene and the lux operon.

19. A recombinant plasmid in accordance with any of Claims 10 through 18 transformed into a host microorganism competent to receive and maintain said plasmid.

20. A recombinant plasmid in accordance with Claim 19 wherein said host microorganism is selected from the group consisting of E. coli, Bacillus, Pseudomonas and Shewanella.

21. A lux operon wherein the luxA and luxB genes are oriented as an operon 5' to 3'.

22. A lux operon according to Claim 21 wherein the lux operon is from X. luminescens.

23. The method of preparing the recombinant plasmid of Claims 10 through 18 comprising a low or multicopy plasmid, an inducible regulatory gene, a transcription terminator, and a substantially promotorless bacterial lux operon that expresses light when induced by said regulatory gene.

24. The method of preparing the recombinant plasmid in accordance with Claim 23 wherein said lux operon is in a plasmid cassette that is reduced in background luminescence by exonuclease digestion in a directed fashion only from the 5' end of the lux operon.

25. The method of preparing the recombinant plasmid in accordance with Claim 24 wherein said lux plasmid cassette is reintroduced into new and complete plasmid vector.

26. The method of preparing the recombinant plasmid in accordance with Claims 24 and 25 transformed into a host microorganism competent to receive and maintain said plasmid.

27. The method of preparing a lux operon wherein the luxA and luxB genes are oriented as an operon 5' to 3'.

28. The method of preparing the lux operon according to Claim 27 wherein the lux operon is from Xenorhabdus luminsecens.

29. The method of preparing the lux operon in accordance with Claim 39 wherein said lux operon contains substantially no. X. luminescens promoter.

30. The method of preparing the lux operon in accordance with Claim 27 wherein said lux operon is reduced in background luminescence by exonuclease digestion in a directed fashion only from the 5' end of the lux operon.

31. The method of preparing the lux operon in accordance with Claim 27 wherein said lux operon is reintroduced into new and complete plasmid vector.

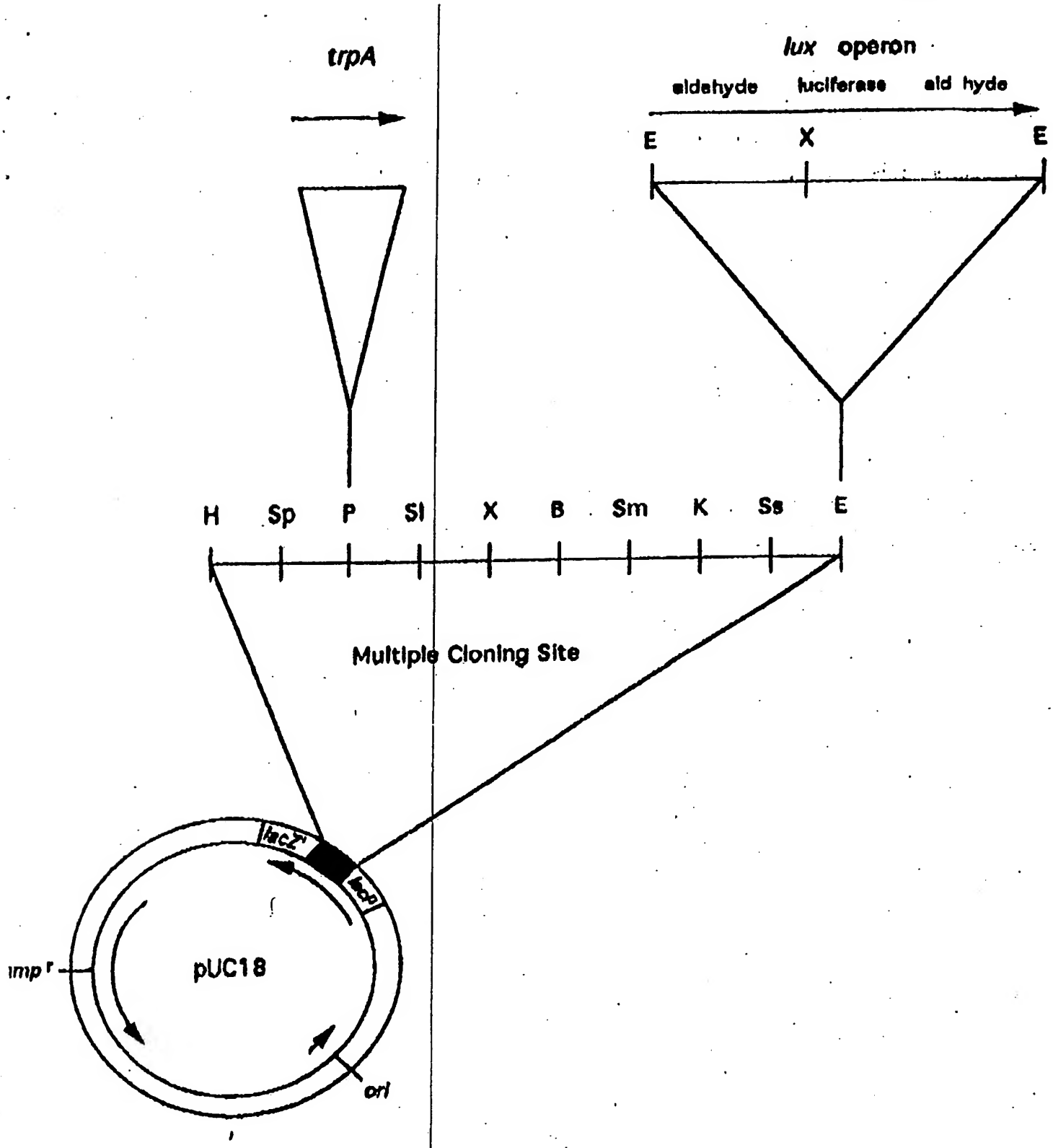
trpA
lux operon
aldehyde
luciferase
ald hyde
E
X
E
H
Sp
P
Sl
X
B
Sm
K
Ss
E
Multiple Cloning Site
lacZ'
lacI
pUC18
amp r
ori

FIG. 1

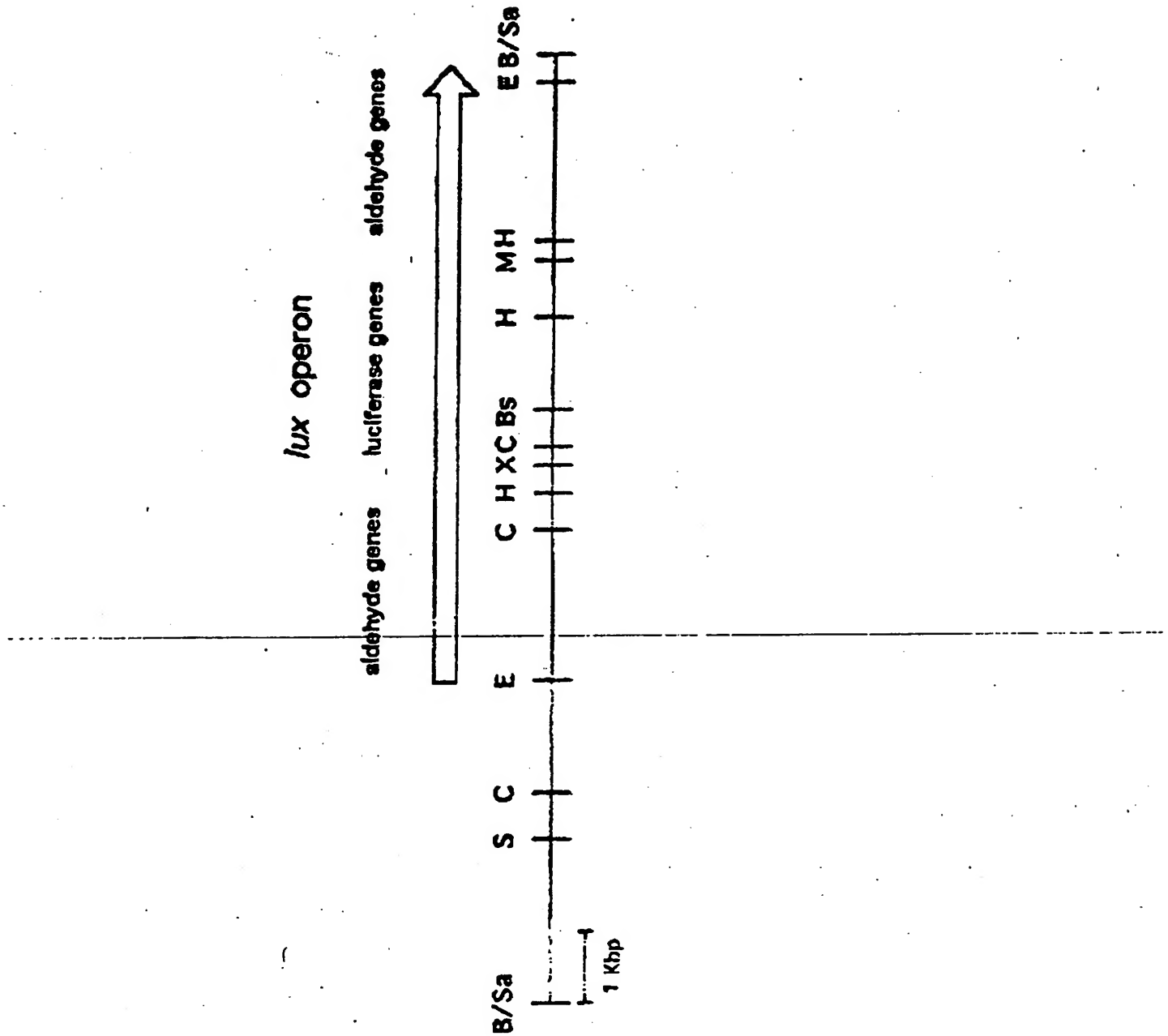
lux operon
aldehyde genes
luciferase genes
aldehyde genes
B/Sa
E
S
C
H
X
C
Bs
H
M
H
E
B/Sa
1 Kbp

FIG. 2

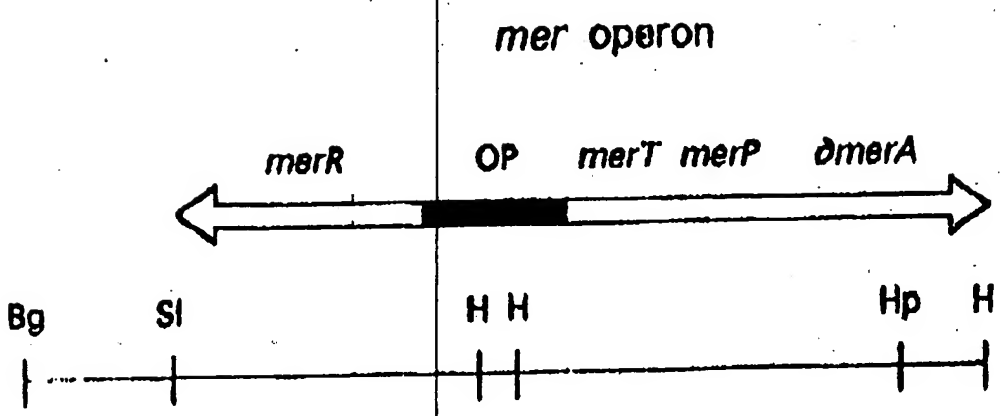

FIG. 3

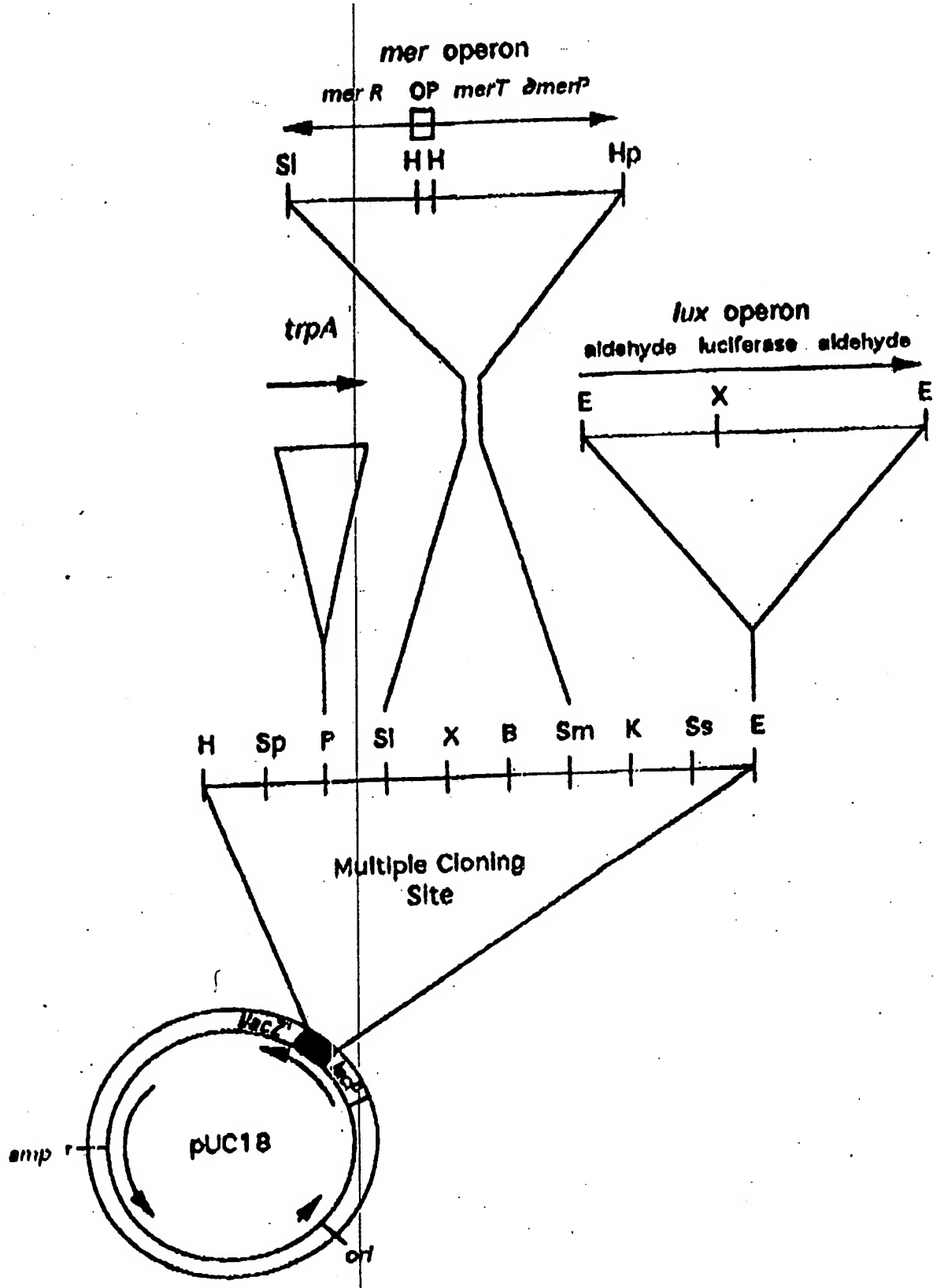
mer operon
merR OP merT ∂merP
Sl
H H
Hp
trpA
lux operon
aldehyde luciferase aldehyde
E
X
E
H Sp P Sl X B Sm K Ss E
Multiple Cloning Site
lacZ'
lacI
amp r
pUC18
ori

FIG. 4

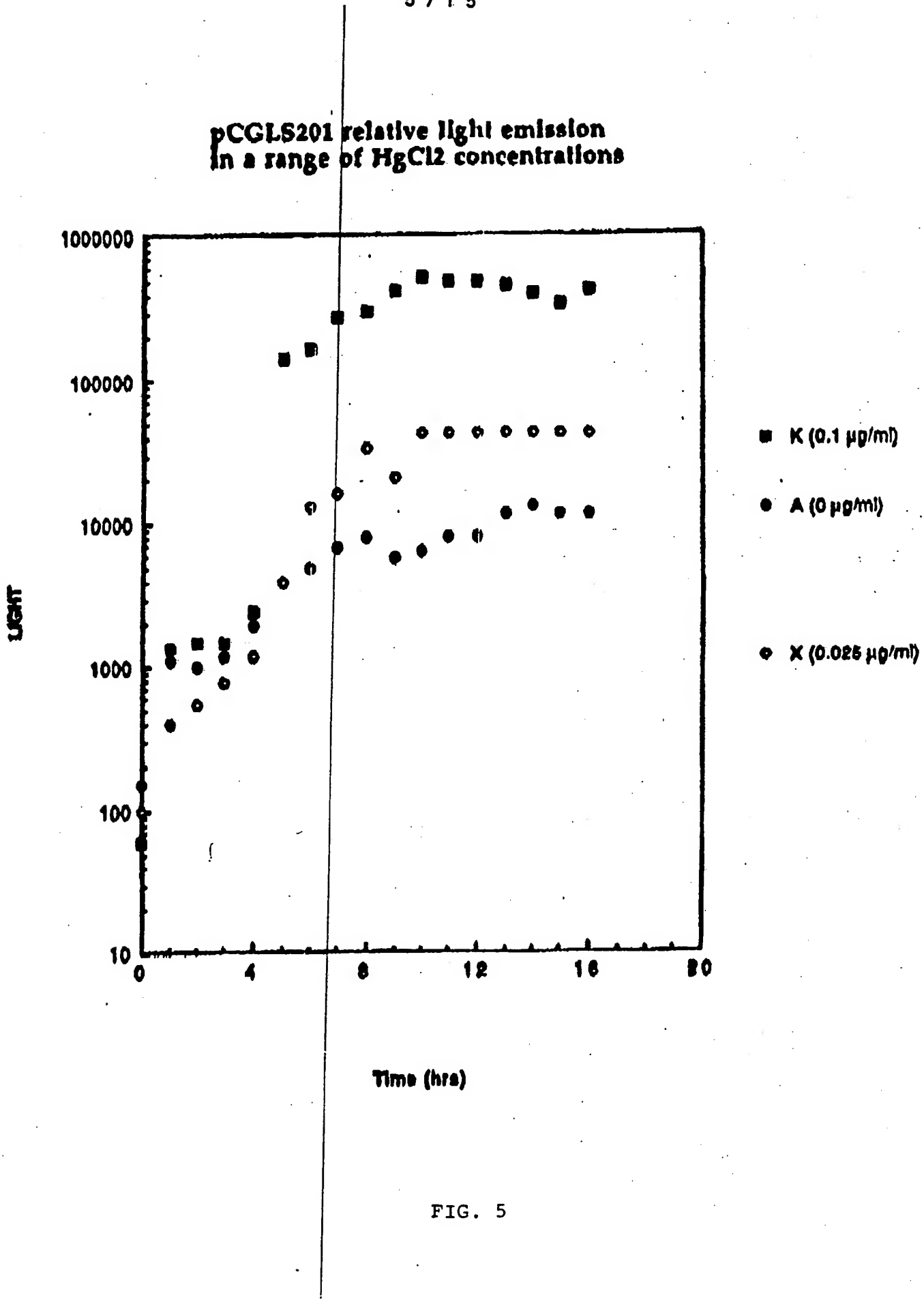
pCGLS201 relative light emission
in a range of HgCl2 concentrations
1000000
100000
10000
1000
100
10
LIGHT
0
4
8
12
16
20
Time (hrs)
K (0.1 µg/ml)
A (0 µg/ml)
X (0.025 µg/ml)

FIG. 5

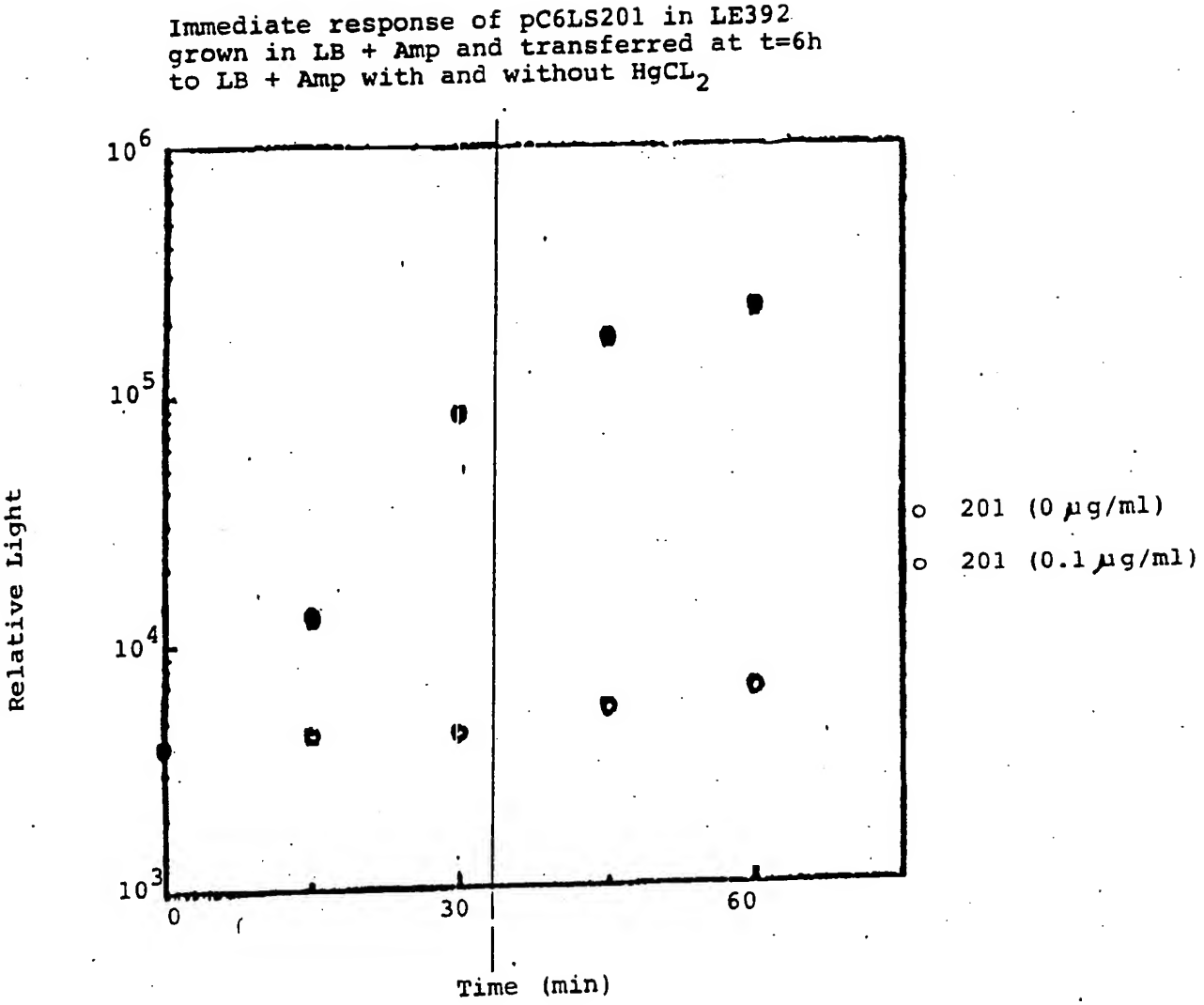
Immediate response of pC6LS201 in LE392 grown in LB + Amp and transferred at t=6h to LB + Amp with and without $HgCL_2$
Relative Light
10^6
10^5
10^4
10^3
0
30
60
Time (min)
o 201 (0 µg/ml)
o 201 (0.1 µg/ml)

FIG. 6

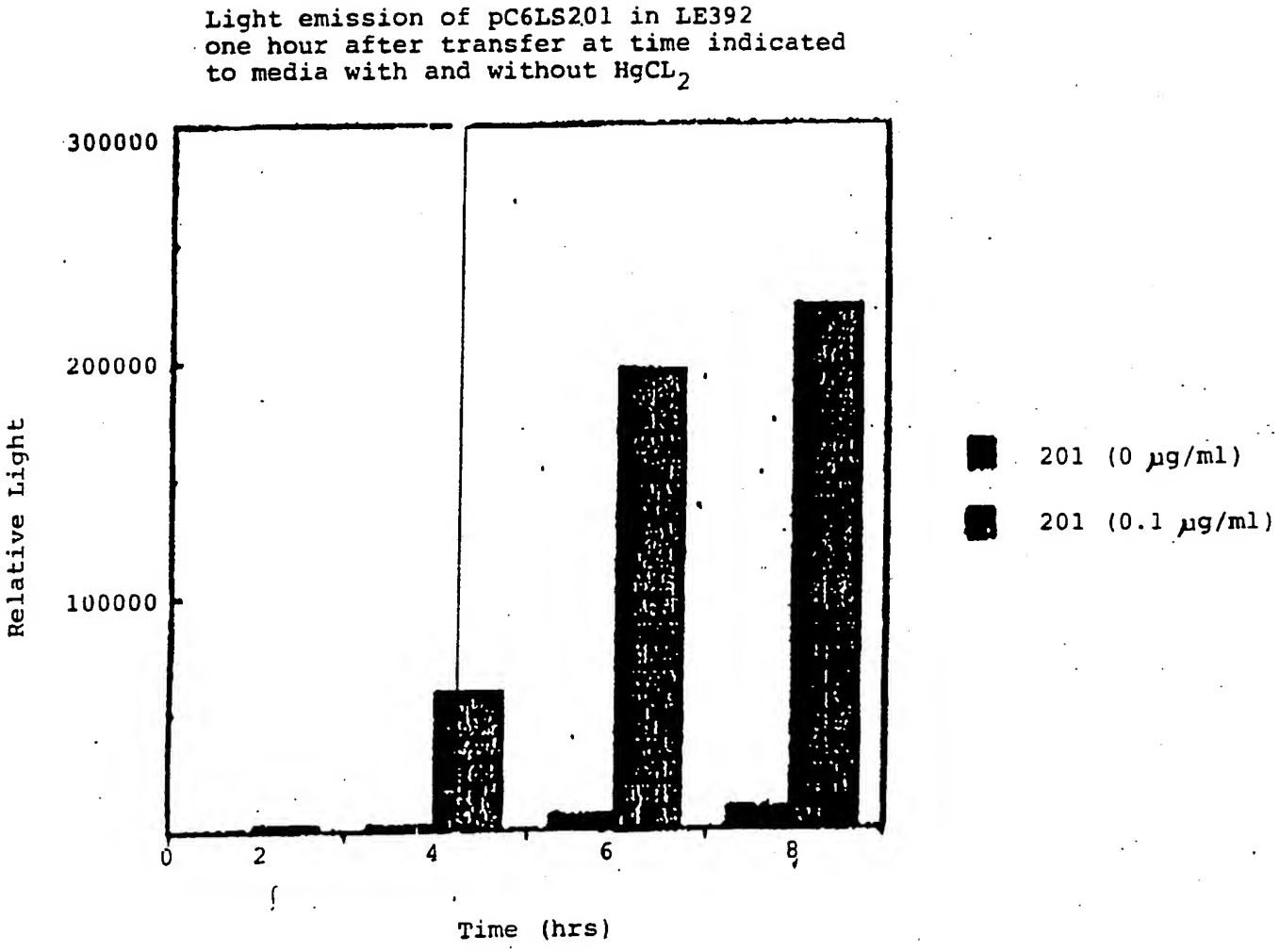
Light emission of pC6LS201 in LE392
one hour after transfer at time indicated
to media with and without $HgCL_2$
Relative Light
300000
200000
100000
0
2
4
6
8
Time (hrs)
201 (0 µg/ml)
201 (0.1 µg/ml)

FIG. 7

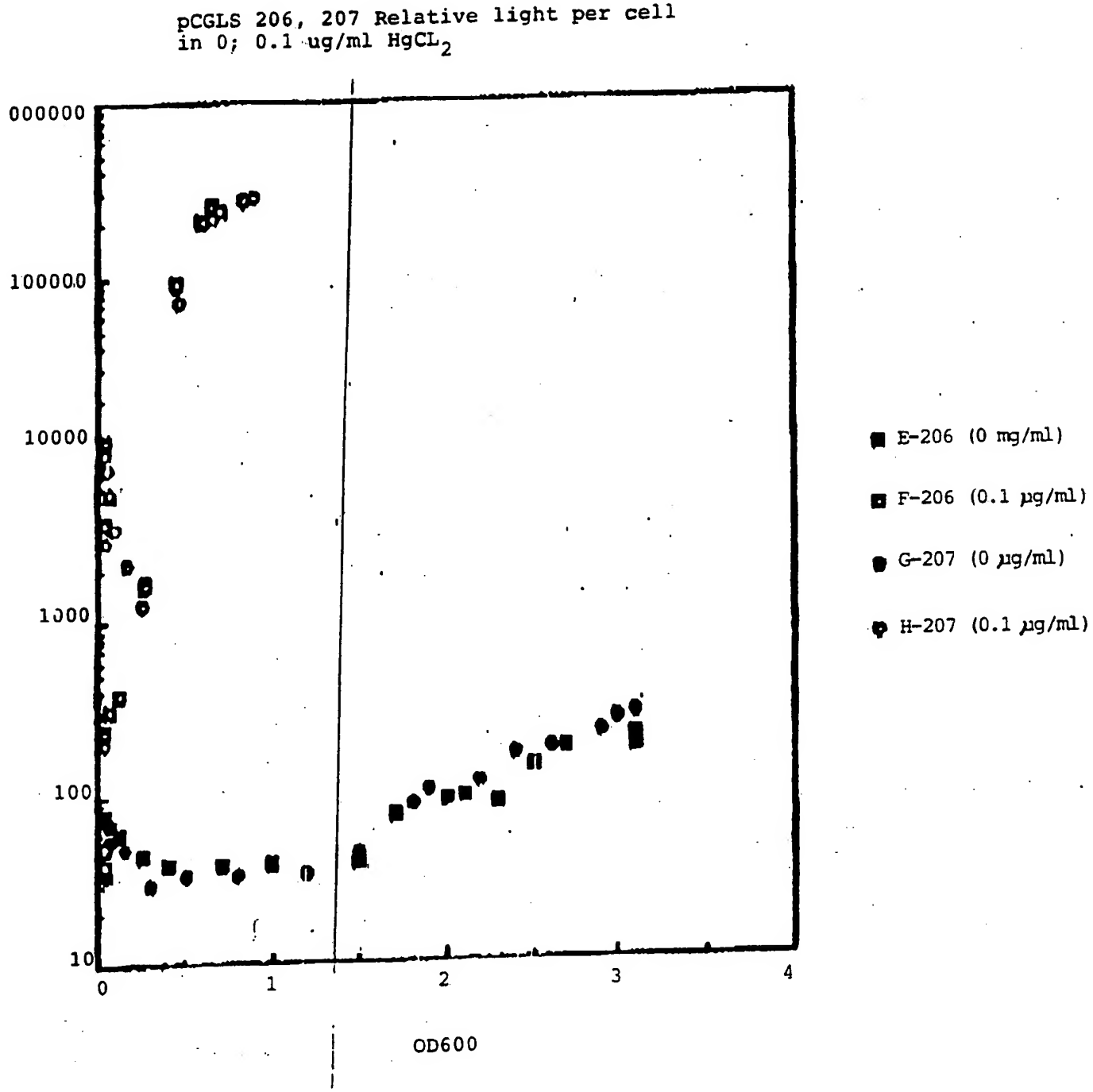
pCGLS 206, 207 Relative light per cell
in 0; 0.1 ug/ml HgCL2
000000
100000
10000
1000
100
10
0
1
2
3
4
OD600
E-206 (0 mg/ml)
F-206 (0.1 µg/ml)
G-207 (0 µg/ml)
H-207 (0.1 µg/ml)

FIG. 8

Examples Of Commercially Available Plasmid Cloning Vehicles

PLASMID	BACTERIAL SYSTEM
pUC18/19	E. coli
pBR322	E. coli
pMK2004	E. coli
pACYC184	E. coli
pLG339	E. coli
pRK353	E. coli
pRK2501	E. coli
pUB110	B. subtilis
pGC2	B. subtilis
pPL531	B. subtilis
pPL608	B. subtilis
pC194	B. subtilis
pK7210	P. aeruginosa/putida
pKT248	P. aeruginosa/putida
pKT230	P. aeruginosa/putida
pFG6	P. aeruginosa/putida
pGU1106	P. aeruginosa/putida
pRO1600	P. aeruginosa/putida
pLAFR33	P. aeruginosa/putida
pHV14	E. coli/B. subtilis
pTE22R	E. coli/B. subtilis
pBS19	E. coli/B. subtilis

FIG. 9

Examples Of Suitable E. coli Strains As Host Carriers

Strains
LE392
C600
DH1
DH5α
DH5αF'I^q
TB1
MC1061
JM103
JM83
JM109
HB101
MM294
MC1061
Y1088
N99
RR1

FIG. 10

Examples Of Suitable Bacillus
and Pseudomonas Strains To Be Used As Host Carriers

Genus/Species	Strains
B. subtilis	JH642
	TKJ5211
	PS6Ø7
	MOØ428
	W168
Pseudomonas putida	KT2442
Pseudomonas aeruginosa	PAØ1

FIG. 11

Examples Of Known Bioluminescent Bact ria With Lux Systems Suitable For Use In Biosensors

	Cloned
Photobacterium phosphoreum	+
Photobacterium leiognathi	+
Vibrio harveyi	+
Vibrio fischeri	+
Xenorhabdus luminescens	+
Vibrio splendidus	-
Vibrio logei	-
Vibrio vulnificus	-
Vibrio cholerae	-
Vibrio orientalis	-
Shewanella hanedai	-

FIG. 12

Summary Of Characterized Metal Systems

Ion	Plasmid or Transposon	Comments
Hg^{2+}	R 100(Tn21)	Gram negative "narrow spectrum"
	pVS1(Tn501)	equivalent to R100
	pDU1358	"broad spectrum"
	p1258	gram positive broad spectrum
	Bacillus sp.	chromosomal; not plasmid
Phenylmercury, mothylmercury and other organomercurials		
	pDU1358	Gram negative; E. coli
	p1258	Gram positive S. aureus and Bacillus
	Bacillus sp.	chromosomal broad spec.
Arsenic system		Gram negative (plasmid R773 in E. coli)
	As(III)	Gram positive (plasmid pI258 in S. aureus and Bacillus
	As(V) arsenate	
	Sb(III)	
	Bi(III)	gratuitous inducer; no resistance to bismuth
Cd^{2+}		S. aureus; Gram positive; cadA, cadB, cadC
$Cd^{2+}/Co^{2+}/Zn^{2+}$		Alcaligenes; Gram negative
Co^{2+}/Ni^{2+}		different plasmid; same Alcaligenes
Chromate		Pseudomonas (not clearly inducible)
		Alcaligenes (clearly inducible; has a second gene)
Ag^{+}		Never thoroughly studied
Ni^{+}		Alcalogenes; Gram negative

FIG. 13

Examples Of Organic
Contaminants Sensed By Biosensors

Phenol

Formaldehyde

PCB's

Trichlorethylene

Pesticides

Octane

Benzene

Toluene

FIG. 14

Recombinant mer Plasmids Derived From 1.4 Kb mer fragment and lux cassette plasmid

lux cassette plasmid	Recombinant mer plasmid	Comments
pCGLS200	pCGLS201	moderate background luminescence
pCGLS204	pCGLS206	dim background luminescence
pCGLS205	pCGLS207	very dim background luminescence

FIG 15

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 92/06361

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12Q1/66; C12Q1/02; C12Q1/18; C12N15/52
G01N21/76; C12N15/70; //H01J43/04H01L31/00

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12Q

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	WO,A,9 004 037 (GENLUX FORSCHUNGSGESELLSCHAFT FÜR BIOLOGISCHE VERFAHREN MBH) 19 April 1990	10-31
Y	see the whole document	1-9

X	WO,A,9 008 836 (GENLUX FORSCHUNGSGESELLSCHAFT FÜR BIOLOGISCHE VERFAHREN MBH) 9 August 1990	10-31
Y	see abstract; claims & DE,A,3 902 982 cited in the application	1-9

Y	US,A,4 863 689 (LEONG ET AL.) 5 September 1989 see abstract	1-9

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° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
26 NOVEMBER 1992	11. 12. 92
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	MOLINA GALAN E.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

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E	WO,A,9 215 687 (VITO) 17 September 1992 see claims	1-31

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. US 9206361 SA 63301

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 26/11/92
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

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EP-A-0496027	29-07-92	None	
WO-A-9215687	17-09-92	None	